

This item was submitted to [Loughborough's Research Repository](#) by the author.  
Items in Figshare are protected by copyright, with all rights reserved, unless otherwise indicated.

## Transmembrane transport of bicarbonate unravelled

PLEASE CITE THE PUBLISHED VERSION

<https://doi.org/10.1002/chem.202100491>

PUBLISHER

Wiley

VERSION

VoR (Version of Record)

PUBLISHER STATEMENT

This is an Open Access Article. It is published by Wiley under the Creative Commons Attribution 4.0 International Licence (CC BY 4.0). Full details of this licence are available at:  
<https://creativecommons.org/licenses/by/4.0/>

LICENCE

CC BY 4.0

REPOSITORY RECORD

Martínez-Crespo, Luis, Sarah Hewitt, Nicola Alessandro De Simone, Vladimír Šindelář, Anthony P Davis, Stephen Butler, and Hennie Valkenier. 2021. "Transmembrane Transport of Bicarbonate Unravelled". Loughborough University. <https://hdl.handle.net/2134/14540631.v1>.

## VIP Transmembrane Transport of Bicarbonate Unravelled\*\*

Luis Martínez-Crespo,<sup>[a]</sup> Sarah H. Hewitt,<sup>[b]</sup> Nicola Alessandro De Simone,<sup>[c]</sup>  
Vladimír Šindelář,<sup>[c]</sup> Anthony P. Davis,<sup>[d]</sup> Stephen Butler,<sup>\*,[b]</sup> and Hennie Valkenier<sup>\*,[a]</sup>

**Abstract:** Anion receptors can be used to transport ions across lipid bilayers, which has potential for therapeutic applications. Synthetic bicarbonate transporters are of particular interest, as defects in transmembrane transport of bicarbonate are associated with various diseases. However, no convenient method exists to directly observe bicarbonate transport and study the mechanisms involved. Here, an assay is presented that allows the kinetics of bicarbonate transport into liposomes to be monitored directly and with great

sensitivity. The assay utilises an encapsulated europium(III) complex, which exhibits a large increase in emission intensity upon binding bicarbonate. Mechanisms involving CO<sub>2</sub> diffusion and the dissipation of a pH gradient are shown to be able to lead to an increase in bicarbonate concentration within liposomes, without transport of the anion occurring at all. By distinguishing these alternative mechanisms from actual bicarbonate transport, this assay will inform the future development of bicarbonate transporters.

## Introduction

The transport of bicarbonate is crucial to many biological processes, such as the regulation of pH<sup>[1,2]</sup> and the removal of metabolic waste.<sup>[3]</sup> The development of synthetic HCO<sub>3</sub><sup>−</sup> transporters could contribute to the study and understanding of various diseases linked to mutations in HCO<sub>3</sub><sup>−</sup> transporting proteins, such as haemolytic anaemia, renal diseases, congenital chloride diarrhoea, and glaucoma.<sup>[3]</sup> Furthermore, HCO<sub>3</sub><sup>−</sup> transporters have potential therapeutic applications and were reported to restore the properties of airway surface liquid in cystic fibrosis airway epithelial tissue.<sup>[4,5]</sup>

Despite the importance of bicarbonate transport in health and disease, most research on synthetic anion transporters to date has focussed on chloride transport.<sup>[6–10]</sup> This is mainly

driven by the ease by which Cl<sup>−</sup> transport can be studied compared to that of HCO<sub>3</sub><sup>−</sup>. Whereas Cl<sup>−</sup> transport through the membranes of liposomes can be readily monitored by fluorescent probes or by ion selective electrodes (ISE),<sup>[8]</sup> no equivalent methods for the study of HCO<sub>3</sub><sup>−</sup> transport exist. The pH sensitive probe HPTS has been widely used to study transport of many different anions and cations;<sup>[11]</sup> however, this method cannot be readily adapted for the study of HCO<sub>3</sub><sup>−</sup> transport, due to the inherent pH variations in HCO<sub>3</sub><sup>−</sup> solutions over time.

Monitoring HCO<sub>3</sub><sup>−</sup> transport across lipid membranes remains a significant challenge. In the homeostasis of biological systems, the transmembrane transport of HCO<sub>3</sub><sup>−</sup> anions and the spontaneous diffusion of CO<sub>2</sub> through membranes are two closely associated processes, which have clearly distinct roles.<sup>[3]</sup> In model systems such as unilamellar vesicles (LUVs), it is not possible to distinguish between the actual transport of HCO<sub>3</sub><sup>−</sup> and mechanisms based on CO<sub>2</sub> diffusion using current assays. Consequently, the exact mechanism(s) by which synthetic HCO<sub>3</sub><sup>−</sup> transporters operate remains ambiguous. Nonetheless, numerous reports on HCO<sub>3</sub><sup>−</sup> transporting anionophores exist,<sup>[12–21]</sup> of which the first described a series of isophthalamides and the natural compound prodigiosin.<sup>[12]</sup> However, indirect methods were employed to study the kinetics of HCO<sub>3</sub><sup>−</sup> transport by these and other compounds (Figure 1). In most cases, either the efflux of Cl<sup>−</sup> out of liposomes was monitored with a chloride selective electrode,<sup>[12–17]</sup> or the influx of Cl<sup>−</sup> was followed with the fluorescent probe lucigenin<sup>[18–20]</sup> or SPQ.<sup>[21]</sup> From the observed Cl<sup>−</sup> transport it was concluded that an antiport process with HCO<sub>3</sub><sup>−</sup> must have taken place. Consequently, these methods are restricted to the study of Cl<sup>−</sup>/HCO<sub>3</sub><sup>−</sup> antiport only, and do not permit the study of exchange with any other anions, nor HCO<sub>3</sub><sup>−</sup> uniport. This limits the possibilities of studying and understanding HCO<sub>3</sub><sup>−</sup> transport.

The only existing method for the direct study of HCO<sub>3</sub><sup>−</sup> transport relies upon <sup>13</sup>C NMR spectroscopy in combination with NaH<sup>13</sup>CO<sub>3</sub> and a paramagnetic species, to distinguish interior from exterior isotopically labelled bicarbonate.<sup>[12–14,21,5]</sup>

[a] Dr. L. Martínez-Crespo, Dr. H. Valkenier  
Université Libre de Bruxelles (ULB), Engineering of Molecular NanoSystems,  
Ecole polytechnique de Bruxelles  
Avenue F.D. Roosevelt 50, CP165/64, 1050 Brussels (Belgium)  
E-mail: hennie.valkenier@ulb.be

[b] Dr. S. H. Hewitt, Dr. S. Butler  
Loughborough University, Department of Chemistry  
Epinal Way, Loughborough, LE11 3TU (UK)  
E-mail: s.j.butler@lboro.ac.uk

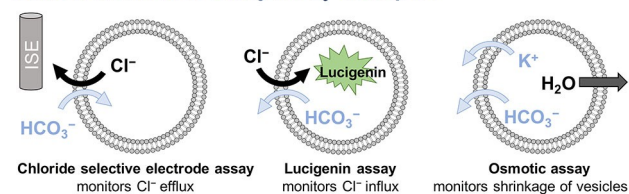
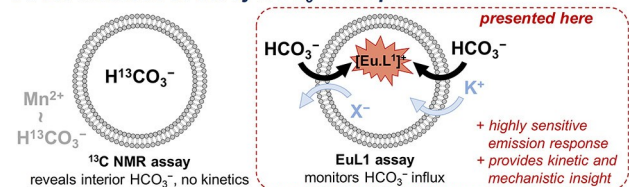
[c] Dr. N. A. De Simone, Prof. V. Šindelář  
Masaryk University, Department of Chemistry and RECETOX, Faculty of  
Science  
Kamenice 5, 625 00 Brno (Czech Republic)

[d] Prof. A. P. Davis  
University of Bristol, School of Chemistry  
Cantock's Close, Bristol, BS8 1TS (UK)

[\*\*] A previous version of this manuscript has been deposited on a preprint server (<https://doi.org/10.26434/chemrxiv.12624425.v3>).

Supporting information for this article is available on the WWW under <https://doi.org/10.1002/chem.202100491>

© 2021 The Authors. Chemistry - A European Journal published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

Indirect methods to study  $\text{HCO}_3^-$  transportDirect methods to study  $\text{HCO}_3^-$  transportFigure 1. Existing and new methods to study  $\text{HCO}_3^-$  transport.

The major disadvantage of this method is the difficulty in monitoring transport processes over time (requiring 3–5 minutes per NMR spectrum), which precludes the accurate measurement of transport kinetics.<sup>[22]</sup> More recently, an osmotic assay was reported where the efflux of  $\text{HCO}_3^-$  by an anionophore is accompanied by the efflux of a cation (by a cationophore), resulting in an osmotic efflux of water, which can be observed as a change in the scattering intensity of the liposome dispersion.<sup>[23]</sup> This is a promising strategy for studying  $\text{HCO}_3^-$  uniport; however, the assay suffers from a low sensitivity and requires relatively large concentrations of transporter to be present in the membranes ( $\sim 10$  mol%).

The limitations associated with current methods clearly call for a new assay that can report on  $\text{HCO}_3^-$  transport directly, accurately and with high sensitivity. Fluorescence-based transport assays offer a great sensitivity and can be readily implemented.<sup>[24,25,26]</sup> A fluorescence-based assay in which the influx of  $\text{HCO}_3^-$  can be monitored directly would thus surmount the current limitations, enabling an accurate comparison and quantification of rates of  $\text{HCO}_3^-$  transport, and verification of the results obtained by indirect methods. Crucially, it would enable the mechanisms of transport to be elucidated unequivocally, including *i)* exchange processes of  $\text{HCO}_3^-$  with different anions (antiport), *ii)* uniport of  $\text{HCO}_3^-$ , and *iii)* identification of actual transport of  $\text{HCO}_3^-$  versus mechanisms based on  $\text{CO}_2$  diffusion. Such an assay requires a water-soluble probe whose emission intensity changes in response to  $\text{HCO}_3^-$  levels, whilst being unaffected by the presence of other anions and cations in the assay.

The cationic europium complex  $[\text{Eu.L}^1]^+$  previously developed by Butler for the purpose of detecting fluoride ions,<sup>[27]</sup> satisfies these requirements.  $[\text{Eu.L}^1]^+$  binds reversibly to  $\text{HCO}_3^-$  in aqueous solution and shows an increase in Eu(III) emission intensity upon binding, particularly within the emission band centred at 615 nm. In contrast,  $[\text{Eu.L}^1]^+$  has negligible responses to  $\text{Cl}^-$  and  $\text{NO}_3^-$  and this made it an ideal candidate for the development of the transport assay. We present here the use of this emissive probe encapsulated in liposomes, to directly

monitor the transport of  $\text{HCO}_3^-$  across the lipid bilayers by fluorescence spectroscopy. We have used this new assay to study  $\text{HCO}_3^-$  transport by a series of highly potent synthetic anionophores (1–3, Figure 2) and natural product prodigiosin (4), for which transport was previously observed indirectly using the lucigenin assay (Figure S1).<sup>[18,20,28]</sup>

This novel  $\text{HCO}_3^-$  assay allows the study of the kinetics and mechanisms of  $\text{HCO}_3^-$  transport by ionophores in unprecedented detail, as well as the comparison of various antiport and uniport processes. We have established that transporters 1–4 operate in different ways, and that only 1 is a “pure”  $\text{HCO}_3^-$  carrier, transporting the anion without interference from other processes. Our results raise the distinct possibility that reported  $\text{HCO}_3^-$  transporters might not transport the  $\text{HCO}_3^-$  anion, but rather dissipate the pH gradient induced by  $\text{CO}_2$  diffusion. The assay represents a significant step forwards for identifying pure  $\text{HCO}_3^-$  transporters and providing the mechanistic insight required to develop their potential biological applications.

## Results and Discussion

## Assay to monitor transport of bicarbonate directly

The cationic Eu(III) complex  $[\text{Eu.L}^1]^+$  (Figure 3c) is based on a cyclen scaffold possessing two pendant quinoline arms that absorb UV light around 330 nm and transfer energy efficiently to the central Eu(III) ion, which emits red light in the range 570–720 nm.<sup>[27]</sup> The Eu(III) probe has an open coordination site, occupied by a single water molecule in aqueous solution which quenches the Eu(III) emission significantly. In the presence of  $\text{HCO}_3^-$ , the coordinated water molecule is displaced upon binding of the hard oxyanion, resulting in a large enhancement in emission intensity (especially around 615 nm) and changes in spectral form (Figure 3a).<sup>[29]</sup> The probe responds to physiologically relevant (millimolar) concentrations of  $\text{HCO}_3^-$  and exhibits high selectivity over poorly coordinating anions that are commonly used in anion transport assays, including  $\text{Cl}^-$  and  $\text{NO}_3^-$ .<sup>[27]</sup> The complex is also sensitive to hydroxide ions, and thus to pH, but this can be controlled readily with the use of a buffer (see ESI).

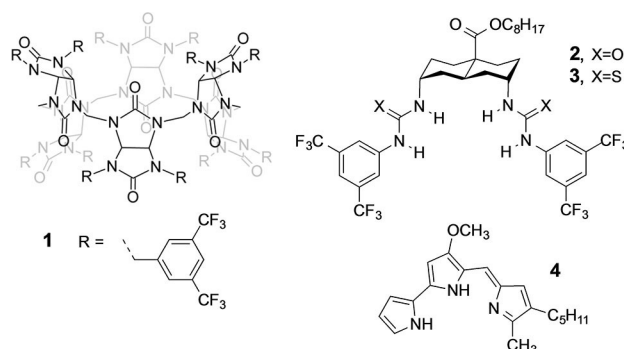
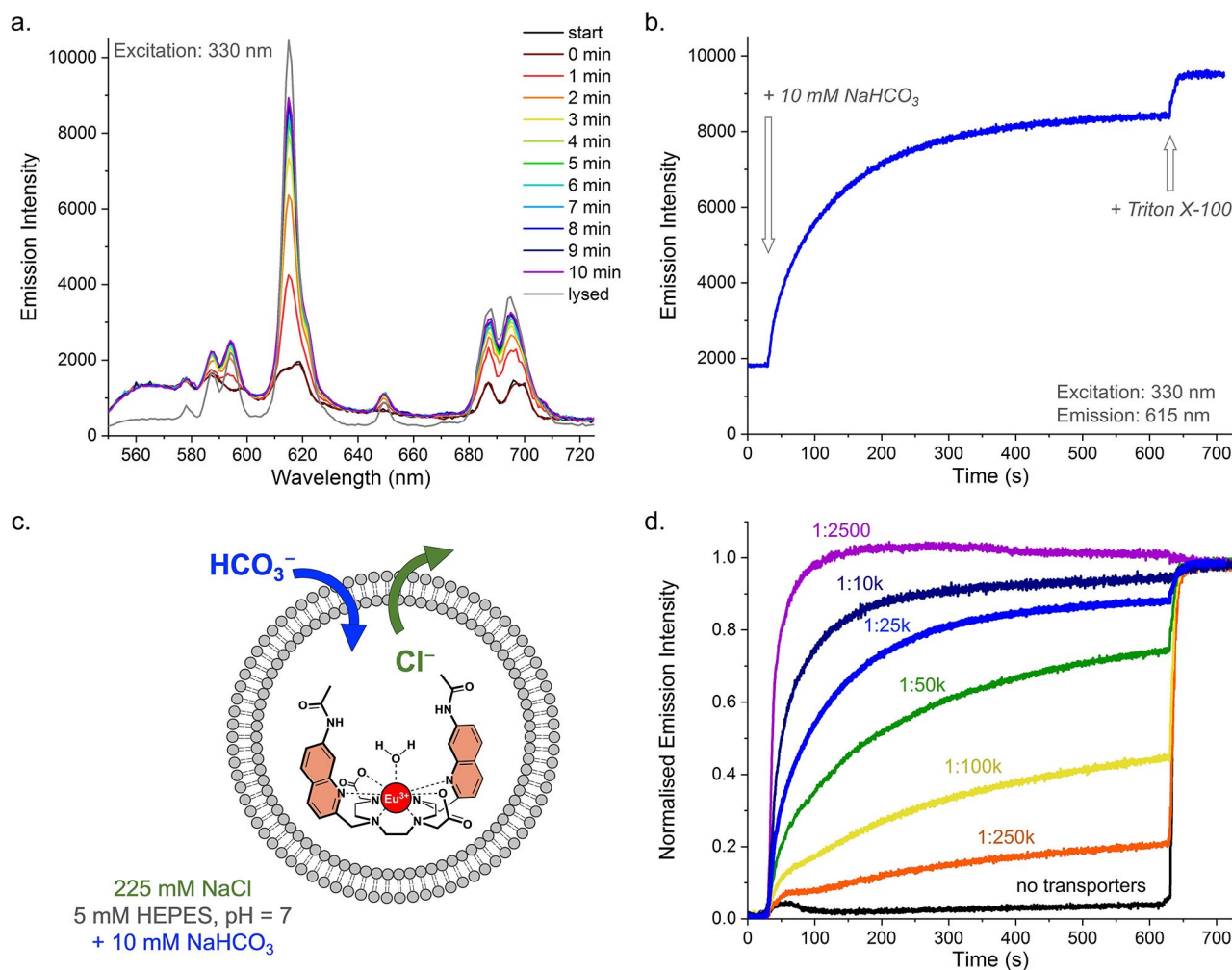


Figure 2. Structures of anionophores 1–4.



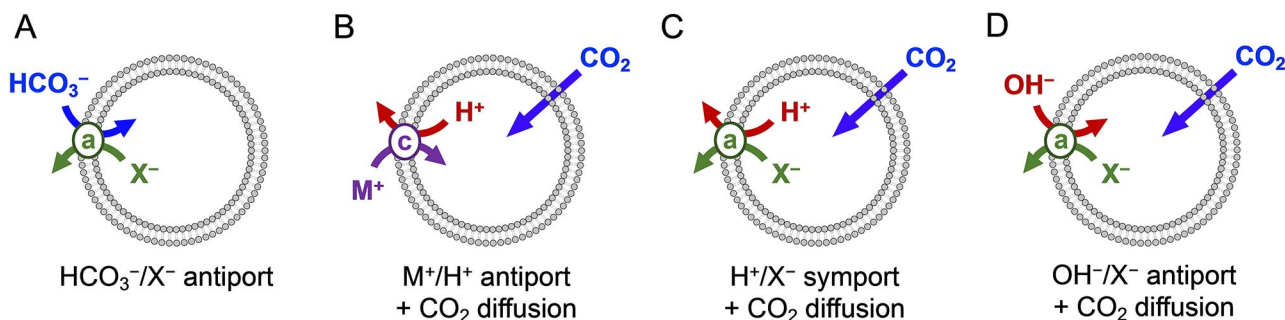
**Figure 3.** Transport of  $\text{HCO}_3^-$  by anionophore 1 preincorporated in LUVs with the probe  $[\text{Eu.L}]^{3+}$  encapsulated (50  $\mu\text{M}$ ), suspended in 225 mM NaCl with 5 mM HEPES at pH 7.0 (interior and exterior), upon addition of 10 mM  $\text{NaHCO}_3$  after 30 seconds and lysis of the LUVs after 10 minutes: a. Emission spectra of  $[\text{Eu.L}]^{3+}$  recorded during the transport by 1 (at 1:25 k transporter to lipid ratio); b. Emission intensity at 615 nm monitored over time for the transport as in a.; c. Schematic representation of EuL1 assay to study transport of  $\text{HCO}_3^-$ ; d. Normalised transport curves for anionophore 1 preincorporated at various anionophore to lipid ratios.

In order to use  $[\text{Eu.L}]^{3+}$  to monitor the transport of  $\text{HCO}_3^-$ , we encapsulated this probe into large unilamellar vesicles (LUVs) consisting of the lipids POPC and cholesterol in a 7:3 ratio and extruded these liposomes through a membrane with 200 nm pores, to obtain LUVs with an average hydrodynamic diameter of 183 nm (Figure S2). Liposomes of this diameter are routinely used for transport experiments by fluorescence spectroscopy and can be prepared reliably with a high degree of unilamellarity, in contrast to much larger vesicles, as used in the  $^{13}\text{C}$  NMR assay.<sup>[12]</sup> An aqueous solution of 225 mM NaCl was present both interior and exterior to facilitate  $\text{HCO}_3^-/\text{Cl}^-$  exchange (antiport), which also contained 5 mM HEPES buffer to adjust the pH to 7.0 (Figure 3c). Anionophore 1 was preincorporated in the membrane of the LUVs and a  $\text{NaHCO}_3$  solution was added to create a  $\text{HCO}_3^-$  concentration gradient of 10 mM (Figure S3). An increase in the intensity of the different emission bands of  $[\text{Eu.L}]^{3+}$  was observed (Figure 3a) upon the addition of  $\text{NaHCO}_3$ . We chose to monitor the  $\Delta J=2$  emission

band around 615 nm (see Figure 3b), as this showed the largest increase, in agreement with observations in titrations of  $[\text{Eu.L}]^{3+}$  with  $\text{HCO}_3^-$ .<sup>[27]</sup> From here on, we will refer to these experimental conditions as the EuL1 assay.

The increase in the emission intensity over time following the addition of  $\text{NaHCO}_3$  indicates that  $\text{HCO}_3^-$  has entered the liposomes. Since hardly any change in the emission intensity was observed in the absence of anionophore 1 (Figure 3d, black curve), we can conclude that bambusuril 1 transports  $\text{HCO}_3^-$  into the liposomes and that the new EuL1 assay allows this process to be monitored. The concentration of 1 was varied (Figure 3d) and a clear increase in the rate of transport was observed for increasing concentrations of anionophore 1. This shows that the EuL1 assay is highly sensitive and can be used to study the kinetics of  $\text{HCO}_3^-$  transport. Furthermore, these results reinforce our previous findings that bambusuril 1 is a very potent  $\text{HCO}_3^-/\text{Cl}^-$  transporter,<sup>[20]</sup> showing activity even at





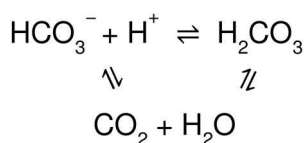
**Figure 4.** Different mechanisms by which apparent transport of  $\text{HCO}_3^-$  could occur. In mechanism A, anionophore (a) exchanges  $\text{HCO}_3^-$  for another anion – we refer to this as actual  $\text{HCO}_3^-$  transport. Mechanisms B–D rely on the diffusion of  $\text{CO}_2$  coupled to transport of  $\text{H}^+$  or  $\text{OH}^-$  by cationophores (c) or anionophores to result in the net increase in  $\text{HCO}_3^-$  concentration, without this anion crossing the membrane.

1:250,000 ratio, which corresponds to 1.6 nM concentration and an average of two bambusurils per LUV.

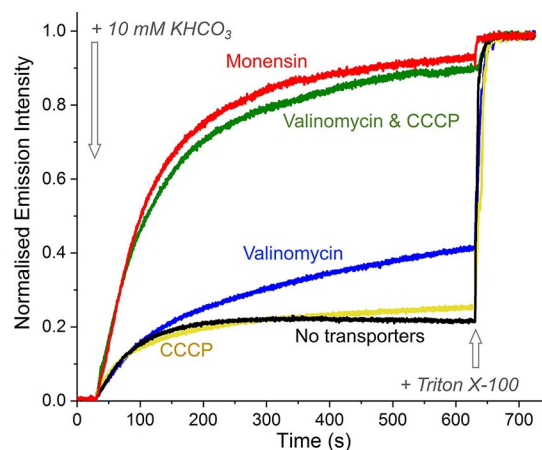
### Differentiating the mechanisms of bicarbonate transport

The processes by which actual and apparent  $\text{HCO}_3^-$  transport can occur are schematically represented in Figure 4. The simplest mechanism for  $\text{HCO}_3^-$  transport is the antiport process with another anion, such as  $\text{Cl}^-$  (Figure 4, mechanism A). However, we should consider that addition of a pulse of  $\text{NaHCO}_3$  to the exterior of the liposomes at  $\text{pH} < 8$  does not only create a gradient of  $\text{HCO}_3^-$ , but also of its conjugate acid  $\text{H}_2\text{CO}_3$ <sup>[23]</sup> and of  $\text{CO}_2$ , formed upon dehydration (Scheme 1).<sup>[30]</sup> At equilibrium the concentration of  $\text{CO}_2$  is almost 1000-fold higher than that of  $\text{H}_2\text{CO}_3$  in aqueous salt solutions.<sup>[31]</sup> Furthermore, it is well known that  $\text{CO}_2$  can diffuse spontaneously across the membranes of cells that play important roles in  $\text{HCO}_3^-$  homeostasis,<sup>[32]</sup> such as red blood cells and renal epithelial cells.<sup>[3]</sup> Upon the addition of the  $\text{HCO}_3^-$  pulse,  $\text{CO}_2$  could thus diffuse across the membranes of our liposomes. This increase in the concentration of  $\text{CO}_2$  inside the liposomes would result in an acidification of the interior, causing a pH gradient to build up, which would stop the diffusion of  $\text{CO}_2$ . However, when transporters that can dissipate pH gradients are present in the membrane, the diffusion of  $\text{CO}_2$  can continue, leading to a net increase in  $\text{HCO}_3^-$  concentration inside the liposomes, without this anion crossing the membrane (Figure 4B–D).

We indeed found that the addition of the cationophore monensin ( $\text{H}^+/\text{M}^+$  antiporter)<sup>[33,34]</sup> to liposomes with  $[\text{Eu.L}^1]^+$  encapsulated gave a clear response upon addition of a  $\text{HCO}_3^-$  pulse (Figure 5, red curve). A similar response was observed



**Scheme 1.** Representation of the interconversion between bicarbonate, carbonic acid, and carbon dioxide.



**Figure 5.** Increase in interior  $\text{HCO}_3^-$  concentration as monitored by the  $\text{Eu.L}^1$  assay in 112 mM  $\text{K}_2\text{SO}_4$  with 5 mM HEPES at  $\text{pH} 7$ , upon addition of 10 mM  $\text{KHCO}_3$  after 30 seconds. Different cation transporters were added to the LUVs at a transporter to lipid ratio of 1:1000.

when the combination of  $\text{K}^+$  transporter valinomycin<sup>[35]</sup> and the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP)<sup>[36]</sup> were added (Figure 5, green curve), while those transporters added individually gave no significant response. Monensin gives similar transport curves in  $\text{MCl}$ ,  $\text{MNO}_3$ , and  $\text{M}_2\text{SO}_4$  solutions (where  $\text{M}^+$  is  $\text{Na}^+$  or  $\text{K}^+$ , see Figure S8 and 6a), in agreement with the anion independent  $\text{CO}_2$  diffusion mechanism B (Figure 4).

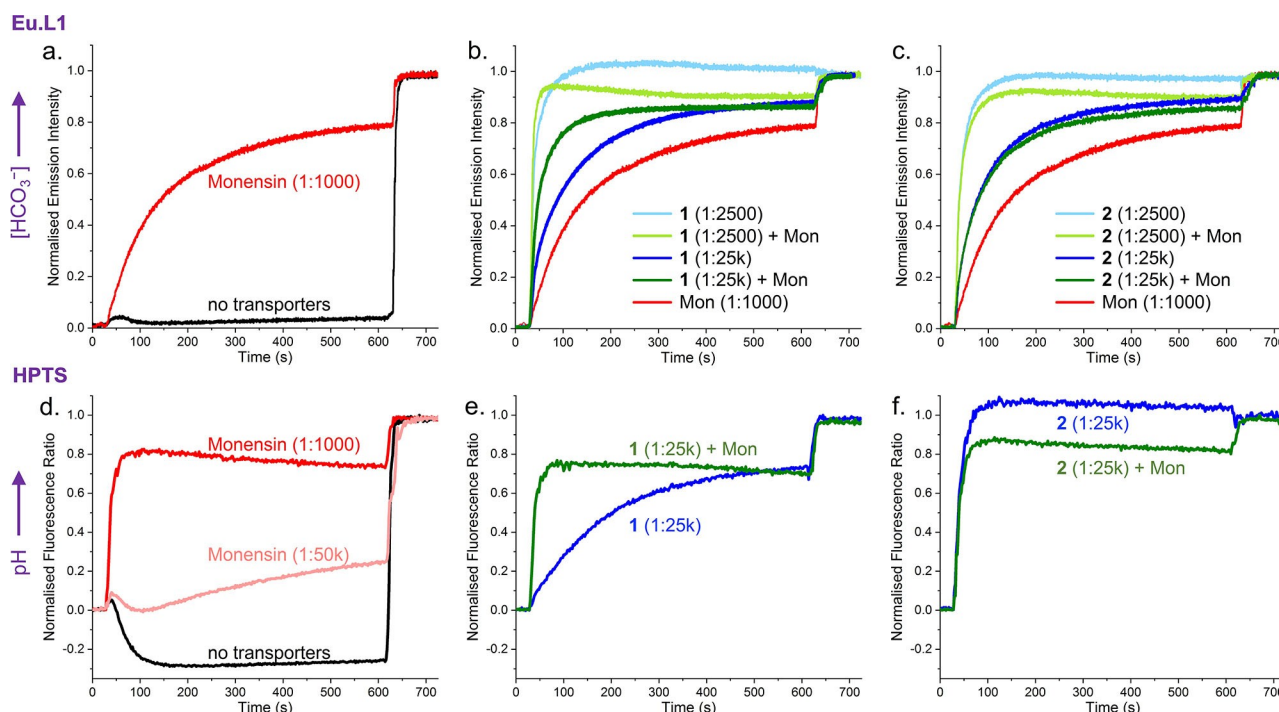
No systematic differences were observed between the experiments using either sodium or potassium salts. This could mean either that monensin performs  $\text{H}^+/\text{Na}^+$  and  $\text{H}^+/\text{K}^+$  antiport at identical rates, or that the formation<sup>[30]</sup> and diffusion of  $\text{CO}_2$  are rate limiting in mechanism B. To distinguish between these possibilities, we varied the concentration of monensin. While decreasing the monensin to lipid ratio from 1:1000 to 1:10,000 gave a lower rate of transport, increasing to a ratio of 1:100 did not significantly impact the rate of transport (Figure S9). This confirms that the diffusion of  $\text{CO}_2$  is rate limiting in the observed increase in  $\text{HCO}_3^-$  concentration within the liposomes and not the  $\text{H}^+/\text{M}^+$  antiport by monensin.

To verify if the pH inside the LUVs changes as expected upon diffusion of  $\text{CO}_2$  (in the absence of ionophores) or upon dissipation of the pH gradient by monensin, transport experiments were performed in which the pH sensitive probe HPTS was encapsulated instead of the bicarbonate sensitive probe  $[\text{Eu.L}]^+$ . All other conditions were identical to those used in the standard EuL1 assay (*i.e.*, 225 mM NaCl, 5 mM HEPES, pH 7.0). The results in Figure 6d show that the addition of 10 mM  $\text{NaHCO}_3$  to LUVs with monensin (1:1000 ratio) results in a rapid increase of the pH to 7.4 (red curve), indicating the equilibration of the pH gradient caused by the addition of the basic solution of  $\text{NaHCO}_3$ . In contrast, addition of  $\text{NaHCO}_3$  to LUVs without transporters results in an acidification of the interior (black curve), in agreement with the formation of carbonic acid upon diffusion of  $\text{CO}_2$ . LUVs with a very low concentration of monensin (1:50,000) show an initial acidification of the interior due to  $\text{CO}_2$  diffusion, followed by a slow increase of the pH due to the  $\text{H}^+/\text{Na}^+$  antiport by monensin. These experiments with HPTS confirm that the apparent transport of  $\text{HCO}_3^-$  by monensin can be attributed to mechanism B. Furthermore, the pH equilibration by monensin at 1:1000 ratio (Figure 6d) is much faster than the apparent  $\text{HCO}_3^-$  transport revealed by the EuL1 assay (Figure 6a), providing further evidence that  $\text{CO}_2$  diffusion (and/or formation<sup>[30]</sup>) is rate limiting in the apparent transport of  $\text{HCO}_3^-$  by monensin (at 1:1000 ratio).

### Determining the transport mechanisms of different anionophores

Next, we studied the  $\text{HCO}_3^-$  transport by anionophores 1–4 in the EuL1 assay in NaCl (blue curves in Figure 6b,c and S12). A clear increase of the emission intensity was observed for all anionophores, even at the relatively low concentration of 1:25,000 (transporter to lipid ratio). A key question we wished to address was whether the observed increase in  $\text{HCO}_3^-$  in the liposomes is due to  $\text{HCO}_3^-/\text{Cl}^-$  antiport transport mechanism A, or rather by  $\text{CO}_2$  diffusion and pH gradient dissipation, as in mechanisms C and D (Figure 4).

Urea and thioureas with acidic N–H groups have been reported to not only transport anions, but also  $\text{H}^+$  (or  $\text{OH}^-$ ), and prodigiosin 4 is a known  $\text{H}^+\text{Cl}^-$  transporter as well.<sup>[37]</sup> Indeed, rapid pH equilibration was observed for anionophores 2–4 (blue curve in Figure 6f and S21). In contrast, bambusurils have an electron deficient cavity formed by twelve polarised methine C–H groups, which can neither be readily deprotonated nor interact strongly with  $\text{OH}^-$ .<sup>[38]</sup> Upon addition of  $\text{NaHCO}_3$  to liposomes with bambusuril 1, a gradual increase in pH was observed, resembling the kinetics of the transport of the basic  $\text{HCO}_3^-$  anion into the LUVs (blue curve in Figure 6e vs 6b). This result concurs with our previous finding that 1 is unable to dissipate pH gradients by  $\text{HCl}$  symport or  $\text{Cl}^-/\text{OH}^-$  antiport.<sup>[20]</sup> This excludes mechanisms C and D for this compound, leaving  $\text{HCO}_3^-/\text{Cl}^-$  antiport (A) as the only possible transport mechanism for 1.



**Figure 6.** Increase in interior  $\text{HCO}_3^-$  concentration as monitored using the EuL1 assay (a–c) or change of the interior pH as monitored using the probe HPTS (d–f) in 225 mM NaCl with 5 mM HEPES at pH 7, upon addition of 10 mM  $\text{NaHCO}_3$  after 30 seconds and lysis of the LUVs 10 minutes after that, to study transport by monensin (a,d), bambusuril 1 (b,e) and urea 2 (c,f). Monensin (1:1000 transporter to lipid ratio) was added to the experiments with anionophores 1 and 2.

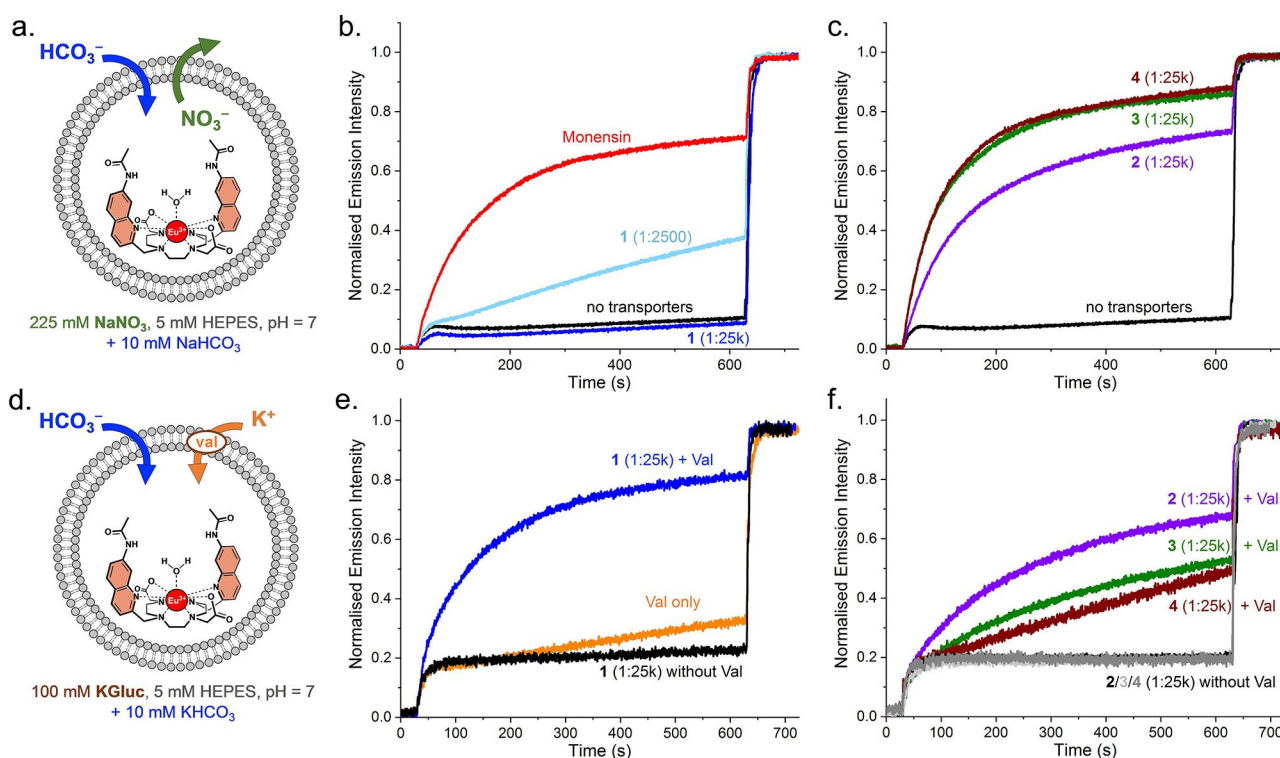
To distinguish the mechanisms involved in the apparent  $\text{HCO}_3^-$  transport by the other compounds, we have made use of the limited rate of  $\text{CO}_2$  diffusion in the EuL1 assay, as observed from the experiments with monensin. For bambusuril **1**, addition of monensin gives a clear increase in the rate of transport as seen from the comparison of the green to the blue curves in Figure 6b and S14. This increase can be understood from the combined effect of mechanism A by **1** and mechanism B by monensin, leading to a higher rate of apparent transport of  $\text{HCO}_3^-$  than by either of these two processes alone. In contrast, addition of monensin to LUVs with anionophores **2–4** did not increase the rate of transport, as shown for **2** in Figure 6c and for **3** and **4** in Figure S12. Because pH equilibration by these compounds is nearly instantaneous (Figure 6f and S21), the addition of a second pathway to dissipate the pH gradient (by monensin) will have no effect, as the overall rate of (apparent)  $\text{HCO}_3^-$  transport will remain limited by  $\text{CO}_2$  diffusion. From this observation we can conclude that anionophores **2–4** primarily act via mechanism C or D.

To test if urea **2** and thiourea **3** can perform any  $\text{HCO}_3^-/\text{Cl}^-$  transport (mechanism A), we have increased the concentrations of **2** and **3** in the membranes of the liposomes to 1:2500 (transporter to lipid ratio). The light blue curves in Figure 6c and S12a show that this ten-fold increase in transporter concentration leads to a significantly faster rate of (apparent)  $\text{HCO}_3^-$  transport, and that this overall rate clearly exceeds rates of transport that are limited by  $\text{CO}_2$  diffusion (as observed in

the curves for monensin  $\geq 1:1000$  ratio, see also Figure S13). From this we can conclude that  $\text{HCO}_3^-/\text{Cl}^-$  antiport mechanism A also takes place. These compounds dissipate the pH gradient faster than they transport  $\text{HCO}_3^-$  and as a result C or D is the main mechanism, up to the point that  $\text{CO}_2$  diffusion becomes rate limiting, after which mechanism A contributes to the apparent  $\text{HCO}_3^-$  transport. It is clear from these data that bambusuril **1** is the only “pure”  $\text{HCO}_3^-$  transporter studied, which functions without interference from other processes.

### Bicarbonate uniport and antiport with nitrate

After demonstrating that our EuL1 assay can distinguish between actual and apparent  $\text{HCO}_3^-$  transport mechanisms, we used the assay to study the exchange of  $\text{HCO}_3^-$  with other anions, or uniport of  $\text{HCO}_3^-$ . Commonly employed indirect methods to study  $\text{HCO}_3^-$  transport rely on the monitoring of  $\text{Cl}^-$  concentrations (Figure 1), preventing their use for studying exchange of  $\text{HCO}_3^-$  and  $\text{NO}_3^-$ , or the uniport of  $\text{HCO}_3^-$ . In contrast,  $[\text{Eu.L}]^+$  can operate in various salt solutions to study other processes than  $\text{HCO}_3^-/\text{Cl}^-$  exchange. Hence, we utilised the EuL1 assay in  $\text{NaNO}_3$  solution, to monitor  $\text{HCO}_3^-/\text{NO}_3^-$  exchange (Figure 7a–c), and in a potassium gluconate (KGluc) solution in the presence of the  $\text{K}^+$  cationophore valinomycin, to study the uniport of  $\text{HCO}_3^-$  (Figure 7d–f).



**Figure 7.** Increase in interior  $\text{HCO}_3^-$  concentration in the presence of anionophores **1–4** monitored by the EuL1 assay in different salt solutions: a–c exchange with nitrate in 225 mM  $\text{NaNO}_3$  with 5 mM HEPES at pH 7, and d–f uniport in 100 mM KGluc with 5 mM HEPES at pH 7 in presence of valinomycin. 10 mM  $\text{NaHCO}_3$  (in b,c) or  $\text{KHCO}_3$  (in e,f) was added after 30 seconds and the LUVs are lysed after 10 minutes. The schematic representations in a and d only show the mechanisms based on actual  $\text{HCO}_3^-$  transport, while mechanisms based on  $\text{CO}_2$  diffusion could also take place.

Compounds **2–4** were found to exhibit efficient (apparent) transport of  $\text{HCO}_3^-$  in  $\text{NaNO}_3$  (Figure 7c) and the rates do not change upon addition of monensin (see Figure S16), similar to the results obtained for these compounds in  $\text{NaCl}$ , indicating that the same combination of mechanisms is occurring. However, bambusuril **1** showed no transport at a 1:25,000 ratio, and only very slow transport was observed when using a 10-fold higher concentration of **1** (1:2500, Figure 7b). This slow  $\text{HCO}_3^-/\text{NO}_3^-$  exchange by **1** resembles previous results reported for  $\text{Cl}^-/\text{NO}_3^-$  exchange, which was found to be 100-fold slower than  $\text{Cl}^-/\text{HCO}_3^-$  exchange by this bambusuril.<sup>[20]</sup> This large difference in  $\text{Cl}^-/\text{HCO}_3^-$  and  $\text{Cl}^-/\text{NO}_3^-$  exchange rates was explained by the very high affinity of **1** for  $\text{NO}_3^-$  ( $K_a = 5 \times 10^{11} \text{ M}^{-1}$  in acetonitrile), which could prevent the release of this anion. In addition, it was proposed that simultaneous binding of a  $\text{Cl}^-$  and a  $\text{HCO}_3^-$  anion in the bambusuril could facilitate the exchange of these anions.<sup>[20]</sup> Even though the formation of an equivalent complex with  $\text{NO}_3^-$  and  $\text{HCO}_3^-$  simultaneously is possible, this does not appear to increase the rate of the exchange of these two anions by **1**. Instead, the very strong binding of  $\text{NO}_3^-$  is the most probable cause for the low rates of  $\text{HCO}_3^-/\text{NO}_3^-$  exchange by **1** (see also Figure S17).

This was further confirmed by the  $\text{HCO}_3^-$  uniport experiment in KGluc (Figure 7e), where  $\text{HCO}_3^-$  was efficiently transported by bambusuril **1**. In this experiment valinomycin transports  $\text{K}^+$  to compensate for the displacement of charge associated to the  $\text{HCO}_3^-$  uniport, while the highly hydrophilic gluconate anion is not readily transported.<sup>[23]</sup> Under these conditions, it is highly unlikely that an anion exchange process takes place and instead bambusuril **1** will have to release the strongly bound  $\text{HCO}_3^-$  and return through the membrane without an anion bound.

In contrast, apparent transport of  $\text{HCO}_3^-$  by thiourea **3** and prodigiosin **4** was much slower when tested in uniport conditions (Figure 7f), compared to in the presence of  $\text{Cl}^-$  or  $\text{NO}_3^-$  (Figure 7c and S12). In  $\text{NaCl}$  and  $\text{NaNO}_3$  the apparent  $\text{HCO}_3^-$  transport by these compounds was mainly attributed to  $\text{H}^+/\text{Cl}^-$  or  $\text{H}^+/\text{NO}_3^-$  cotransport in combination with  $\text{CO}_2$  diffusion (mechanism C, or equivalent mechanism D). The poor rates of transport in KGluc indicate that these compounds are less efficient protonophores ( $\text{H}^+$  or  $\text{OH}^-$  transporters) than  $\text{H}^+/\text{Cl}^-$  or  $\text{H}^+/\text{NO}_3^-$  cotransporters and that they are not efficient  $\text{HCO}_3^-$  uniporters either. This result corroborates with other reports in which prodigiosin **4** was found to be a poor protonophore.<sup>[37]</sup> The rate of apparent  $\text{HCO}_3^-$  transport by urea **2** in KGluc is higher than those observed for **3** and **4** and the increase in the rate of transport with a higher concentration of **2** (Figure S18) indicates that **2** is able to perform actual  $\text{HCO}_3^-$  uniport (see SI for further mechanistic discussions). Nonetheless, bambusuril **1** is clearly the most efficient  $\text{HCO}_3^-$  uniporter tested.

### Quantification of transport rates

To verify the qualitative trends and comparisons described above, we fitted the transport data from the EuL1 assay with

single and double exponential functions, to obtain half-lives and initial rates respectively (see SI for details). Due to the slight differences observed in the equilibration levels of the different transport curves after normalisation and the effect of pH on the emission levels (see SI for a discussion), half-lives are more reliable to compare transport data, as these values indicate the time required to reach half of the final transport level and are thus a measure of how fast equilibrium is reached, independent of absolute emission values. The obtained values for the half-lives are given in Table 1 (see Table S1 for additional data). In  $\text{NaCl}$ , the comparison of half-lives of transport by **1** with and without monensin clearly shows that equilibrium is reached much faster in the presence of monensin (Table 1 and Figure S15), confirming the additivity of mechanisms A and B as discussed above. In contrast, the half-lives for **2–4** are nearly identical in the presence and absence of monensin, both in  $\text{NaCl}$  and in  $\text{NaNO}_3$ .

Table 1 also shows that the overall half-lives obtained from the apparent  $\text{HCO}_3^-$  transport by anionophores **1–4** (in absence of monensin) are rather similar in  $\text{NaCl}$ . However, the different pH profiles could affect this comparison (see SI Section 2.10) and it would thus be better to compare the different transporters in the presence of monensin. Under those conditions,  $\text{CO}_2$  diffusion-based mechanisms contribute to the transport for all the compounds, but as this process has a limited and thus constant rate, the differences in half-lives between anionophores **1–4** (in presence of monensin) can be attributed to the differences in rates of  $\text{HCO}_3^-/\text{Cl}^-$  antiport (mechanism A) by the anionophores. In this comparison, bambusuril **1** is clearly the most active ionophore for  $\text{HCO}_3^-/\text{Cl}^-$  antiport. Bis-urea **2** and bis-thiourea **3** show similar rates of transport and are slightly

**Table 1.** Performance of anionophores **1–4** in the EuL1 assay.

Salt	Anionophore	Concentration (anionophore: lipid)	Half-life (s) <sup>[a]</sup> without monensin	Half-life (s) <sup>[a]</sup> with monensin
NaCl	None		*	82
	<b>1</b>	1:2500	10	4
	<b>1</b>	1:25 k	64	21
	<b>2</b>	1:2500	12	11
	<b>2</b>	1:25 k	51	50
	<b>3</b>	1:2500	12	11
	<b>3</b>	1:25 k	46	47
	<b>4</b>	1:25 k	59	74
NaNO <sub>3</sub>	None		*	81
	<b>1</b>	1:2500	*	67
	<b>1</b>	1:25 k	*	85
	<b>2</b>	1:2500	45	40
	<b>2</b>	1:25 k	89	85
	<b>3</b>	1:2500	16	14
	<b>3</b>	1:25 k	65	59
	<b>4</b>	1:25 k	61	68
KGluc <sup>[b]</sup>	None		*	124
	<b>1</b>	1:25 k	83	38
	<b>2</b>	1:25 k	140	45
	<b>3</b>	1:25 k	180	42
	<b>4</b>	1:25 k	*	n.d.

[a] Calculated from a single exponential fit of the transport curve, see ESI for details. [b] Transport in KGluc was studied in presence of valinomycin. \* Transport was absent or too slow to quantify. n.d. = not determined.



more active than prodigiosin **4**, for which the half-life is very close to that of transport by monensin alone.

In contrast, in  $\text{NaNO}_3$ , transport by bambusuril **1** was too slow to be quantified, while addition of monensin resulted in half-lives that were identical or slightly lower than for monensin alone (for the lower and higher concentration of **1**, respectively). Half-life values also indicate that apparent  $\text{HCO}_3^-$  transport in  $\text{NaNO}_3$  by **2** is a bit slower than in  $\text{NaCl}$ , and that this is not the case for **3** and **4**, which show similar rates in both salt solutions.

The values of half-lives obtained in KGluc and in presence of valinomycin clearly demonstrate that **1** is a much better  $\text{HCO}_3^-$  uniporter than any of the other transporters. Again, transport by **1** can be enhanced by adding both valinomycin and monensin due to an additivity of mechanisms. In contrast, while rates of **2** and **3** in presence of both valinomycin and monensin are faster than with valinomycin only (Table 1), these rates are the same as with monensin only (see Table S2), indicating that these compounds could perform  $\text{HCO}_3^-/\text{H}^+$  symport (see SI for further discussion).

### Comparison of the EuL1 assay with existing methods

Our EuL1 assay for monitoring  $\text{HCO}_3^-$  transport directly overcomes numerous disadvantages of existing methods, while combining their advantages, to offer a complementary tool in anion transport research (Figure 1). The only other direct  $\text{HCO}_3^-$  transport assay is based on  $^{13}\text{C}$  NMR spectroscopy,<sup>[12]</sup> which suffers from low sensitivity and poor time resolution. Furthermore, we observed that the cationophore monensin also gives a positive response in the  $^{13}\text{C}$  NMR assay for  $\text{HCO}_3^-/\text{Cl}^-$  transport (Figure S22), which demonstrates that this assay cannot distinguish between  $\text{CO}_2$  diffusion-based mechanisms and actual  $\text{HCO}_3^-$  transport. Indirect assays, such as ISE and lucigenin assays,<sup>[12–20]</sup> have not been able to provide mechanistic insights, nor allow comparisons between various  $\text{HCO}_3^-$  antiport and uniport processes. The osmotic  $\text{HCO}_3^-$  uniport assay is the only method reported so far that showed diffusion of neutral  $\text{HCO}_3^-$ -based species in combination with  $\text{H}^+$  transport by monensin,<sup>[23]</sup> in agreement with our findings. However, the drawbacks of the osmotic assay are the very high concentrations of ionophores required due to the low sensitivity and the lower compatibility of the assay with preincorporated lipophilic transporters in the membrane during the preparation of the LUVs.

We have exploited the attractive features of the EuL1 assay to discover that bambusuril **1** can efficiently perform  $\text{HCO}_3^-/\text{Cl}^-$  antiport and  $\text{HCO}_3^-$  uniport, while bisurea **2**, thiourea **3**, and prodigiosin **4** mainly combine  $\text{CO}_2$  diffusion and pH gradient dissipation, leading to *apparent*  $\text{HCO}_3^-$  transport. Compounds **1–4** have previously been shown to act as  $\text{HCO}_3^-$  transporters in the lucigenin assay<sup>[18,20]</sup> and prodigiosin **4** also in the ISE and  $^{13}\text{C}$  NMR assays.<sup>[12]</sup> However, those experiments could not distinguish between actual and apparent transport of  $\text{HCO}_3^-$  anions. Notably, most of the  $\text{HCO}_3^-$  transporters reported in the literature resemble compounds **2–4** and can transport  $\text{H}^+$  or

$\text{OH}^-$ .<sup>[37]</sup> This transport activity combined with  $\text{CO}_2$  diffusion could be the mechanism of apparent  $\text{HCO}_3^-$  transport for many reported compounds. It is striking that selectivity for transport of  $\text{Cl}^-$  and  $\text{NO}_3^-$  over  $\text{HCO}_3^-$  has been reported for only two compounds, a biotinuril macrocycle and a bis-triazole,<sup>[39,40]</sup> which do not have acidic protons and are thus likely to be poor  $\text{H}^+$  and  $\text{OH}^-$  transporters.

Our results imply that only for compounds that show apparent  $\text{HCO}_3^-$  transport without dissipating pH gradients (such as **1**) and for very potent anion transporters for which the rate of total apparent  $\text{HCO}_3^-$  transport surpasses the limited rate of  $\text{CO}_2$  diffusion (**2** and **3**), we can conclude with certainty that these can act as actual anionophores for  $\text{HCO}_3^-$ . This should be taken into account in the future development of  $\text{HCO}_3^-$  anionophores and can be readily verified with the EuL1 assay.

### Conclusion

We have developed a new assay to directly monitor the transport of  $\text{HCO}_3^-$  into liposomes by fluorescence spectroscopy, using the encapsulated europium complex  $[\text{Eu.L}^1]^+$  that provides a luminescence increase upon binding  $\text{HCO}_3^-$ . This assay provides a rapid and highly sensitive signal that enables anion transport kinetics to be determined and low concentrations of anionophores to be used. By combining anionophores with monensin in this direct and sensitive assay, it was possible to distinguish *actual transport* of  $\text{HCO}_3^-$  anions from alternative mechanisms based on  $\text{CO}_2$  diffusion, which lead to an increase of  $\text{HCO}_3^-$  concentration in the liposomes without this anion crossing the membrane.

Our assay provides unprecedented insight into the mechanisms of  $\text{HCO}_3^-$  transport by anionophores and leads to the conclusion that we should doubt if many of the reported  $\text{HCO}_3^-$  transporters are actually capable of transporting this anion, or that they rather operate by dissipating the pH gradient resulting from  $\text{CO}_2$  diffusion. Furthermore, the versatility of the assay compared to all existing assays was demonstrated by comparing  $\text{HCO}_3^-/\text{Cl}^-$  and  $\text{HCO}_3^-/\text{NO}_3^-$  antiport and  $\text{HCO}_3^-$  uniport processes for the first time.

We are convinced that the new opportunities provided by this assay to study transport of  $\text{HCO}_3^-$  efficiently and in new mechanistic detail will contribute to the further development of  $\text{HCO}_3^-$  transporters for biomedical purposes, such as channel replacement therapies.<sup>[6,41]</sup> As the anion binding properties of lanthanide probes such as  $[\text{Eu.L}^1]^+$  can be tuned for different anions,<sup>[29,42]</sup> the assay developed in this work could also be readily adapted to study transport of other anions. Furthermore, our work will also inform the future design of Eu(III) probes capable of monitoring spatio-temporal  $\text{HCO}_3^-$  dynamics within living cells. Indeed, a structurally related Eu(III) complex has already been shown to enter living cells and localise to specific subcellular compartments.<sup>[43]</sup> This feature, combined with the long luminescence lifetime of  $[\text{Eu.L}^1]^+$  and its derivatives augurs well for cellular imaging of  $\text{HCO}_3^-$  transport with high signal-to-noise, using time-gated fluorescence microscopy.

## Acknowledgements

The results reported here are part of a project that has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (Grant agreement No. 802727). H.V. is a research associate of the Fonds de la Recherche Scientifique – FNRS. L.M.C. and H.V. also thank the ULB, “Fonds Van Buuren” and “Fonds Defay” for grants that enabled the purchase of the fluorescence spectrometer. S.J.B. and S.H.H. acknowledge the support of the EPSRC (EP/S032339/1) and the Wellcome Trust (204500/Z/16/Z). V.Š. thanks the Czech Science Foundation (No. 20-13922S).

## Conflict of Interest

The authors declare no conflict of interest.

**Keywords:** bicarbonate · fluorescent probes · ion transport · membranes · supramolecular chemistry

- [1] M. Tresguerres, J. Buck, L. R. Levin, *Pfluegers Arch. Eur. J. Physiol.* **2010**, *460*, 953–964.
- [2] A. Gorbatenko, C. W. Olesen, E. P. Boedtker, S. F. P. Pedersen, *Front. Physiol.* **2014**, *5*, 130.
- [3] E. Cordat, J. R. Casey, *Biochem. J.* **2009**, *417*, 423–439.
- [4] A. Gianotti, V. Capurro, L. Delpiano, M. Mielczarek, M. García-Valverde, I. Carreira-Barral, A. Ludovico, M. Fiore, D. Baroni, O. Moran, R. Quesada, E. Caci, *Int. J. Mol. Sci.* **2020**, *21*, 1488.
- [5] K. A. Muraglia, R. S. Chorghade, B. R. Kim, X. X. Tang, V. S. Shah, A. S. Grillo, P. N. Daniels, A. G. Cioffi, P. H. Karp, L. Zhu, M. J. Welsh, M. D. Burke, *Nature* **2019**, *567*, 405–408.
- [6] A. P. Davis, D. N. Sheppard, B. D. Smith, *Chem. Soc. Rev.* **2007**, *36*, 348–357.
- [7] I. Alfonso, R. Quesada, *Chem. Sci.* **2013**, *4*, 3009–3019.
- [8] H. Valkenier, A. P. Davis, *Acc. Chem. Res.* **2013**, *46*, 2898–2909.
- [9] A. Vargas Jentzsch, A. Hennig, J. Mareda, S. Matile, *Acc. Chem. Res.* **2013**, *46*, 2791–2800.
- [10] J. T. Davis, P. A. Gale, R. Quesada, *Chem. Soc. Rev.* **2020**, *49*, 6056–6086.
- [11] N. Sakai, S. Matile, *J. Phys. Org. Chem.* **2006**, *19*, 452–460.
- [12] J. T. Davis, P. A. Gale, O. A. Okunola, P. Prados, J. C. Iglesias-Sánchez, T. Torroba, R. Quesada, *Nat. Chem.* **2009**, *1*, 138–144.
- [13] N. Busschaert, P. A. Gale, C. J. E. Haynes, M. E. Light, S. J. Moore, C. C. Tong, J. T. Davis, J. William, A. Harrell, *Chem. Commun.* **2010**, *46*, 6252–6254.
- [14] N. J. Andrews, C. J. E. Haynes, M. E. Light, S. J. Moore, C. C. Tong, J. T. Davis, W. A. Harrell Jr, P. A. Gale, *Chem. Sci.* **2011**, *2*, 256–260.
- [15] E. Hernando, V. Soto-Cerrato, S. Cortés-Arroyo, R. Pérez-Tomás, R. Quesada, *Org. Biomol. Chem.* **2014**, *12*, 1771–1778.
- [16] C. Cossu, M. Fiore, D. Baroni, V. Capurro, E. Caci, M. Garcia-Valverde, R. Quesada, O. Moran, *Front. Pharmacol.* **2018**, *9*, 852.
- [17] M. Olivari, R. Montis, S. N. Berry, L. E. Karagiannidis, S. J. Coles, P. N. Horton, L. K. Mapp, P. A. Gale, C. Caltagirone, *Dalton Trans.* **2016**, *45*, 11892–11897.
- [18] S. Hussain, P. R. Brotherhood, L. W. Judd, A. P. Davis, *J. Am. Chem. Soc.* **2011**, *133*, 1614–1617.
- [19] W. A. Harrell Jr, M. L. Bergmeyer, P. Y. Zavalij, J. T. Davis, *Chem. Commun.* **2010**, *46*, 3950–3952.
- [20] H. Valkenier, O. Akrawi, P. Jurček, K. Sleziačková, T. Lízal, K. Bartík, V. Šindelář, *Chem* **2019**, *5*, 429–444.
- [21] P.-Y. Liu, S.-T. Li, F.-F. Shen, W.-H. Ko, X.-Q. Yao, D. Yang, *Chem. Commun.* **2016**, *52*, 7380–7383.
- [22] While standard NMR instrument configurations do not permit to monitor the kinetics of  $\text{H}^{13}\text{CO}_3^-$  transport, ref [5] shows that it is not impossible.
- [23] L. A. Jowett, E. N. W. Howe, X. Wu, N. Busschaert, P. A. Gale, *Chem. Eur. J.* **2018**, *24*, 10475–10487.
- [24] A. Barba-Bon, Y.-C. Pan, F. Biedermann, D.-S. Guo, W. M. Nau, A. Hennig, *J. Am. Chem. Soc.* **2019**, *141*, 20137–20145.
- [25] K. M. Bąk, B. van Kolck, K. Masłowska-Jarzyna, P. Papadopoulou, A. Kros, M. J. Chmielewski, *Chem. Commun.* **2020**, *56*, 4910–4913.
- [26] N. Renier, O. Reinaud, I. Jabin, H. Valkenier, *Chem. Commun.* **2020**, *56*, 8206–8209.
- [27] S. J. Butler, *Chem. Commun.* **2015**, *51*, 10879–10882.
- [28] H. Valkenier, L. W. Judd, H. Li, S. Hussain, D. N. Sheppard, A. P. Davis, *J. Am. Chem. Soc.* **2014**, *136*, 12507–12512.
- [29] S. E. Bodman, S. J. Butler, *Chem. Sci.* **2021**, *12*, 2716–2734.
- [30] C. Ho, J. M. Sturtevant, *J. Biol. Chem.* **1963**, *238*, 3499–3501.
- [31] A. L. Soli, R. H. Byrne, *Mar. Chem.* **2002**, *78*, 65–73.
- [32] V. Endeward, M. Arias-Hidalgo, S. Al-Samir, G. Gros, *Membranes* **2017**, *7*, 61.
- [33] R. Sandeaux, J. Sandeaux, C. Gavach, B. Brun, *Biochim. Biophys. Acta Biomembr.* **1982**, *684*, 127–132.
- [34] Y. N. Antonenko, T. I. Rokitskaya, A. Huczyński, *Biochim. Biophys. Acta Biomembr.* **2015**, *1848*, 995–1004.
- [35] P. J. F. Henderson, J. D. McGivan, J. B. Chappell, *Biochem. J.* **1969**, *111*, 521–535.
- [36] S. G. McLaughlin, J. P. Dilger, *Physiol. Rev.* **1980**, *60*, 825–863.
- [37] X. Wu, L. W. Judd, E. N. W. Howe, A. M. Withecombe, V. Soto-Cerrato, H. Li, N. Busschaert, H. Valkenier, R. Pérez-Tomás, D. N. Sheppard, Y.-B. Jiang, A. P. Davis, P. A. Gale, *Chem* **2016**, *1*, 127–146.
- [38] T. Lízal, V. Šindelář, *Isr. J. Chem.* **2018**, *58*, 326–333.
- [39] M. Lisbjerg, H. Valkenier, B. M. Jessen, H. Al-Kerdi, A. P. Davis, M. Pittelkow, *J. Am. Chem. Soc.* **2015**, *137*, 4948–4951.
- [40] S. Chen, S. Zhang, C. Bao, C. Wang, Q. Lin, L. Zhu, *Chem. Commun.* **2016**, *52*, 13132–13135.
- [41] R. Quesada, R. Dutzler, *J. Cystic Fibrosis* **2020**, *19*, S37–S41.
- [42] S. H. Hewitt, G. Macey, R. Mailhot, M. R. J. Elsegood, F. Duarte, A. M. Kenwright, S. J. Butler, *Chem. Sci.* **2020**, *11*, 3619–3628.
- [43] R. Mailhot, T. Traviss-Pollard, R. Pal, S. J. Butler, *Chem. Eur. J.* **2018**, *24*, 10745–10755.

Manuscript received: February 8, 2021

Version of record online: May 1, 2021